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1. **Exp. Allergy (1997) 27(8): 932-41.**

2. **J. Allergy Clin. Immunol (1997) 99(3): 374-384.**

3. **J. Immunol (1993) 150(3): 1047-54.**

4. **Int. Arch. Allergy Appl. Immunol. (1990) 92(3): 226-32.**

5. **Int. Arch. Allergy Immunol. (1992) 99(2-4): 380-1.**

Thanks,
"Neon" Phuong N. Huynh
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Immunol gic charact rizati n of monoclonal antibodi s that m dulat human IgE binding t the major birch pollen allergen Bet v 1

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Rudolf Valenta, MD[b] Dardilly and Paris, France, and Vienna, Austria

Background: Bet v 1 and homologous proteins represent major allergens for almost 95% of patients allergic to tree pollen and approximately 70% of those allergic to fruits and vegetables. As yet, no continuous (sequential) IgE epitopes have been determined for Bet v 1, and evidence has accumulated that Bet v 1 IgE epitopes belong to the conformational (discontinuous) type.
Objective: A panel of 85 mouse monoclonal anti-Bet v 1 antibodies was raised as a tool with which to study the interaction of human IgE antibodies with Bet v 1.
Methods: The epitopes of selected monoclonal antibodies (mAbs) were characterized by mapping with synthetic overlapping peptides and by cross-competition experiments. Cross-reactivity of Bet v 1–specific mAbs with tree and plant food allergens was investigated by Western blotting. The influence of Bet v 1–specific mAbs on the IgE–Bet v 1 interaction was studied by competition assays with immobilized purified recombinant Bet v 1 and by basophil histamine release experiments.
Results: Antibodies that increased the IgE binding to Bet v 1 up to fivefold could be defined, whereas others inhibited IgE binding to Bet v 1 up to 99% and competed with the Bet v 1–induced histamine release from patients' basophils.
Conclusion: The activity of the enhancing antibodies is interpreted as a stabilization of Bet v 1 states/IgE epitopes, which are either more accessible for certain IgE antibodies or are recognized with higher affinity. Those mAbs that competed with the Bet v 1–IgE interaction, if humanized or produced as recombinant antibody fragments, might be considered as potential tools for local allergy therapy. (J Allergy Clin Immunol 1997;99:374-84.)

Key words: Monoclonal antibodies, major birch pollen allergen Bet v 1, modulation of IgE binding

IgE-mediated allergic reactions such as rhinitis, conjunctivitis, and bronchial asthma represent a health problem of increasing importance for more than 20% of the population in industrialized countries.[1] The imme-

Abbreviations used	
BSA:	Bovine serum albumin
mAb:	Monoclonal antibody
OD:	Optical density
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide electrophoresis
TBS-B-T:	20 mmol/L Tris, 150 mmol/L NaCl, 1% wt/vol BSA, 0.05% vol/vol Tween-20

diate release of biologic mediators, such as histamine, from mast cells and basophils results from the cross-linking of FcεRI receptor-bound specific IgE by allergens. Current immunologic attempts to control allergic reactions include hyposensitization therapy,[2,3] competition of IgE binding to allergic effector cells,[4] and antagonism of IL-4 effects.[5] The rapid progress in the molecular characterization of allergens, however, makes it tempting to consider strategies for interference with the allergen–IgE antibody interaction, especially when patients are sensitized to only a few dominant allergens.[6]

In the case of tree pollen, more than 95% of the patients with allergy are sensitized to the major birch pollen allergen Bet v 1, and 60% of these patients react exclusively to Bet v 1.[7] Bet v 1 is a 17 kd protein, which is highly homologous with pathogenesis-related plant proteins[8] and has been shown to possess RNase activity.[9] IgE antibodies from patients allergic to birch pollen cross-react with homologous allergens from trees of the Fagales order[10-13] (alder, hazel, hornbeam) and related fruit and vegetable allergens,[14-16] thus explaining IgE-mediated food allergy observed in a significant percentage of patients allergic to birch pollen, which may lead to clinical food allergy. Although for other major allergens continuous (sequential) IgE epitopes could be defined,[17] no Bet v 1–derived IgE-binding peptide or recombinant fragment has been found as yet, suggesting that IgE epitopes of Bet v 1 belong to the discontinuous (conformational) type. For these reasons, the interaction of patients' IgE antibodies with Bet v 1 was investigated by using a panel of 85 Bet v 1–specific mouse monoclonal antibodies (mAbs). Among the mAbs, several that enhanced IgE binding to Bet v 1 up to fivefold could be

identified, whereas thers reduced IgE binding to Bet v 1 up t 99% and inhibited Bet v 1–induced specific histamine release.

METHODS

Generation of mouse mAbs against Bet v 1

Inbred BALB/c mice were immunized intraperitoneally with 1 mg of pollen grains from *Betula alba* (Sigma Chemical Co., St Louis, Mo.) and were emulsified in Freund's complete adjuvant on day 0 and in Freund's incomplete adjuvant on day 15. Spleens were removed for fusion 3 days after a final intravenous injection of 0.5 mg of pollen grains in 0.5 ml of phosphate-buffered saline (PBS). Hybridization was carried out by using the nonsecreting myeloma cell line SP2/0-Ag8, with polyethylene glycol 1000 (Sigma) as fusing agent. Hybrid cells were placed in 96-well Falcon tissue plates (Becton Dickinson) and fed with Dulbecco's modified eagle medium F12 (Gibco BRL, Bethesda, Md.) supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml), glutamine (2 mmol/L), 10% vol/vol horse serum (Gibco), 1% ADCM (Additif de Culture; Blood Transfusion Center, Lyon, France), 10^{-5} mol/L azaserine (Sigma), and 5×10^{-5} mol/L hypoxanthine. Hybridoma supernatants were harvested after 6 days and screened by ELISA for secretion of antibodies against birch pollen proteins. Cells from positive wells were cloned by the limiting dilution method. Aliquots of positive cell clones were expanded for 6 days and cryopreserved and propagated in ascitic fluid from BALB/c mice that had received one intraperitoneal injection of 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane, Sigma) 15 days before. Approximately 10^5 hybridoma cells in 1 ml of PBS were injected intraperitoneally; and 10 days later, ascitic fluid was collected from each mouse.

Purification and labeling of the mAbs

After centrifugation of the ascitic fluid, the antibody fraction was isolated by ammonium sulfate precipitation and anion-exchange chromatography on a Zephyr-D Silicium column (IBF Sepracor, Villeneuve la Garenne, France) equilibrated with 20 mmol/L Tris HCl, pH 8. Proteins were eluted with an NaCl gradient (ranging from 0 to 1 mol/L NaCl). Two-milliliter fractions were collected and tested by ELISA for reactivity to birch pollen extract. The fractions containing antibodies reacting with Bet v 1 were pooled, membrane-dialyzed, and frozen. Aliquots of purified mAbs were peroxidase-labeled according to the method of Wilson and Nakane[18]. Peroxidase-labeled mAbs were diluted in 50% glycerol and stored at –20° C.

Preparation of birch pollen extracts

Pollen extracts were prepared from *Betula pendula* (Smith Kline Beecham Pharmaceutical, London, U.K.) and *Betula alba* (Sigma) as previously described[19] with slight modifications. Briefly, 10 gm of pollen grains was added to 200 ml of distilled water and agitated overnight at 4° C with a magnetic stirrer. After centrifugation (3500 g for 15 minutes), the supernatant was precipitated with 72% wt/vol $(NH_4)_2SO_4$ for 1 hour. The pellet was then resuspended in 20 ml of PBS and dialyzed against polyethylene glycol 35000 (Sigma) by using dialysis membranes with a cutoff of 2 kd (Sepracor IBF) to concentrate the proteins. A centrifugation at 60,000 g for 30 minutes eliminated the particles. Final extracts were diluted to 50% with glycerol and stored in aliquots of 1 ml at –20° C. Total protein concentration of the birch pollen extract was estimated by the absorption at 280 nm of the solution and by using the Bio-Rad DC Protein Assay kit (Bio-Rad, Richmond, Calif.). The protein

concentrations of *B. pendula* and *B. alba* pollen extracts were estimated around 1 mg/ml and 10 mg/ml, respectively. To further characterize the extracts, 15% sodium dodecylsulfate (SDS)–acrylamide gels were used with O'Farrell/Laemmli Tris-Glycine-SDS buffer (Buffer EZE formula 2; Kodak, Rochester, N.Y.). Natural birch pollen proteins were reduced with 2-β-mercaptoethanol, boiled for 3 minutes, loaded at 2 μg/cm, and subjected to electrophoresis. Several dilutions of the pollen extract and known amounts of low-molecular-weight protein standards were loaded on the same gel. After completion of the run, the gel was silver-stained, and a visual estimation of the concentration of Bet v 1 was made by comparison with the molecular weight standards. It was estimated that Bet v 1 represented about 10% to 20% of the proteins in the extract.

Purification of natural Bet v 1 by affinity chromatography

Three milligrams of purified mAb 7 was coupled per milliliter of Affi-Gel Hz Hydrazide agarose gel (Bio-Rad) according to the manufacturer's instructions. One milliliter of birch pollen extract was incubated with 2 ml of immunosorbent overnight at 4° C. After two washes with PBS and one wash with PBS 1 mol/L NaCl, the proteins were eluted with 0.1 mol/L glycine-HCl and 0.1 mol/L NaCl, pH 2.5; and the eluted fractions were immediately neutralized with 1 mol/L Tris, pH 8.0. The fractions were checked by capture ELISA for their Bet v 1 content; and Bet v 1–enriched fractions were pooled, diluted to 50% with glycerol, and stored at –20° C. Purified Bet v 1 was analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue (BioRad) staining. The protein content of immunoaffinity-purified natural Bet v 1 was estimated by absorption at 280 nm by using the molecular extinction coefficient of 2.0, predicted from the amino acid composition of the allergen.

Direct ELISA

The *B. pendula* pollen extract was diluted 1:200; pollen extracts of alder, oak, hazel, willow, plane, beech, and elm (Smith Kline Beecham Pharmaceuticals) were diluted 1:50 in coating buffer (carbonate buffer: pH 9.6, 15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$). This diluted solution was coated onto the wells of 96-microwell ELISA plates (Immunoplate Maxisorp F96 certified; Nunc, Rosklide, Denmark) overnight at room temperature. The plates were then washed manually once with washing buffer (PBS, 0.05% Tween-20). One hundred microliters of ascitic fluid or purified antibodies diluted 1:100 in dilution buffer (TBS-B-T buffer: 20 mmol/L Tris, 150 mmol/L NaCl, 1% wt/vol bovine serum albumin [BSA], 0.05% vol/vol Tween-20) was added per well and incubated for 2 hours at 37° C, then washed once as described above. One hundred-microliter aliquots of sheep anti-mouse IgG coupled to horseradish peroxidase (Biosys, Compiègne, France) was added to each well at 500 ng/ml dilution in TBS-B-T buffer. The antibodies were incubated for 2 hours at 37° C. Wells were then washed three times with washing buffer. One hundred microliters of peroxidase substrate, 2,2' azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diluted to 1 mg/ml in citrate-phosphate buffer was then added to each well, and the colorimetric reaction was read at 405 nm. Background was determined as the optical density (OD) of control wells without mouse antibodies.

Sandwich ELISA for th quantificati n of Bet v 1

Among the anti-Bet v 1 antibodies, mAb 8 and peroxidase-labeled mAb 10 were selected to present and to analyze Bet v

1 in a sandwich ELISA, respectively. Ascitic fluid of mAb 8 was diluted 1:1500 in carbonate buffer and coated onto the wells of 96-microwell ELISA plates overnight at room temperature. Plates were then washed once with washing buffer. One hundred microliters of birch pollen extract or of purified Bet v 1 diluted in TBS-B-T buffer was added to each well. After 2 hours of incubation at 37° C, the plates were washed once. One hundred microliters of peroxidase-labeled mAb 10 diluted to 0.05 µg/ml in TBS-B-T buffer was added to each of the wells and incubated for 2 hours at 37° C. After three washes, the ELISA was read as described above. Background was determined as the optical density (OD) of wells without birch pollen.

Binding of mouse mAbs to nitrocellulose-blotted Bet v 1 and related allergens

Different allergen extracts (birch pollen, alder pollen, hazel pollen, or hazelnut and apple) and recombinant Bet v 1[20] were separated by SDS-PAGE[21] and immunoblotted[22] so that approximately 50 ng/cm Bet v 1 or the homologous allergens was present on the membrane. Nitrocellulose strips containing the major allergen of each extract or recombinant Bet v 1 were cut (each row represents strips from one sheet) and incubated with the mAbs diluted 1:100. Bound mouse IgG was detected with an iodine 125–labeled sheep anti-mouse antiserum diluted 1:500 (Amersham, Buckinghamshire, U.K.). Bip 1, another mouse mAb specific for Bet v 1, which has been previously described,[23] was used as positive control.

Immunoprecipitation of radioiodinated Bet v 1

Natural Bet v 1 was iodinated with the Iodo Beads kit (Pierce, Rockford, Ill.). Fifty microliters of purified mAb diluted in PBS-BSA 1% was incubated for 45 minutes at room temperature with ^{125}I-labeled birch pollen extract (20,000 cpm, diluted in PBS–1% BSA) in a 5 ml polypropylene tube (Nunc). Fifty microliters of a dilution (15 ml beads diluted to 50 ml in PBS–1% BSA) of protein G coupled to Sepharose 4B (Sigma) was added to each tube and incubated for 45 minutes at room temperature. The Sepharose was then centrifuged for 5 minutes at 2500 g and washed three times with 1 ml of PBS. After the last centrifugation, the radioactivity bound to the pellet corresponding to the complexes ^{125}I-Bet v 1–IgG anti-Bet v 1 was counted in a Wizard gamma-counter (Wallac Oy, Turku, Finland). The pellet was resuspended in O'Farrell/Laemmli Tris-Glycine-SDS buffer and loaded on a 15% SDS-polyacrylamide gel, which was dried and exposed to a Kodak XOMAT film (Kodak, Heidelberg, Germany). The precipitation was also done with an IgG₁ mouse mAb without specificity for Bet v 1 (negative control).

Cross-inhibition of mAbs in ELISA

An ELISA as described above was used, except that revelation with a suboptimal concentration of peroxidase-labeled mAb was preceded by incubations (2 hours at room temperature) with 100 molar excess of competitor mAbs. Inhibition = {(M − i) ÷ (M − b) × 100, where M is the OD measured in the absence of inhibitor, i is the OD measured in the presence of 100 molar excess of mAb competitor, and b is the OD measured in the absence of mAb.

Epitope mapping of Bet v 1–specific mAbs with overlapping peptides

Dodecapeptides with three–amino acid overlap were synthesized according to the Bet v 1 sequence[8] (Chiron, Victoria,

Australia). Approximately 50 ng of each peptide was coated to ELISA plates (Nunc). Bound anti-Bet v 1 mAbs were detected with an alkaline phosphatase–coupled rabbit anti-mouse immunoglobulin antiserum (Dianova, Hamburg, Germany) and subsequent analysis of the color reaction in an ELISA reader (Dynatech, Denkendorf, Germany).

Selection and characterization of patients allergic to Bet v 1

Human sera and basophils were obtained from patients allergic to birch pollen with a clinical history suggestive of tree pollen allergy (allergic rhinitis, conjunctivitis, or asthma), a positive skin prick test response to birch pollen extract, and a positive RAST for birch pollen. The IgE reactivity profile of sera was established by using recombinant Bet v 1 as previously described.[24]

Modulation of patients' IgE binding to recombinant Bet v 1 by mAbs

The influence of mAbs on the binding of allergic patients' IgE antibodies to Bet v 1 was studied by using nitrocellulose-blotted or dot-blotted purified recombinant Bet v 1. Nitrocellulose strips containing recombinant Bet v 1 (approximately 5 to 10 ng/cm) were cut from the same preparative sheet, saturated in buffer A (50 mmol/L Naphosphate, pH 7.5, 0.5% Tween-20, 0.5% BSA, 0.05% NaN₃), and preincubated with different Bet v 1–specific mAbs, sera without specificity for Bet v 1, or buffer. Strips were then washed and incubated with serum IgE (diluted 1:5) from patients allergic to Bet v 1. Bound IgE was detected with ^{125}I-labeled monoclonal anti-human IgE antibodies (Pharmacia, Uppsala, Sweden). After washing, strips were exposed to x-ray films (Kodak, Heidelberg, Germany), and signals were analyzed by scanning with a densitometer (Hirschmann, Unter-haching, Germany). The same assay was done with undenatured Bet v 1 (10 ng/dot), which was dot-blotted to nitrocellulose; and 56 sera from patients allergic to birch pollen were tested. Quantification of inhibition or enhancement of IgE binding to Bet v 1 in the case of the dot blot experiment was determined by counting of the nitrocellulose in a gamma counter (Wallac).

Histamine release assay from basophils

Tris-albumin buffer (Tris-A) containing 25 mmol/L Tris (pH 7.6), 5 mmol/L KCl, 130 mmol/L NaCl, and human serum albumin at 0.33 mg/ml was used for the washing of the cells.[25] The same buffer supplemented with 0.6 mmol/L Ca²⁺ and 1 mmol/L Mg²⁺ (Tris-ACM) was used in histamine release experiments.

Six milliliters of glucose-dextran was added to 30 ml of heparinized blood from a patient allergic to Bet v 1. Erythrocytes and platelets were allowed to sediment for 45 minutes at room temperature. The peripheral blood lymphocytes, including basophils, were recovered in the plasma supernatant. After centrifugation, the cells were washed twice in Tris-A buffer and resuspended in Tris-ACM buffer. Fifty microliters of purified natural Bet v 1 at 0.1 ng/ml was incubated with 2 × 10⁶ cells for 40 minutes at 37° C in a final volume of 500 µl. In all experiments a control for the determination of the spontaneous histamine release was included. Incubation was stopped by the addition of 500 µl of cold PBS. Eight hundred fifty–microliter aliquots of the cell suspension (for determination of the total histamine content) or of the supernatants were recovered and acidified with 100 µl of 4N perchloric acid. After 20 minutes of
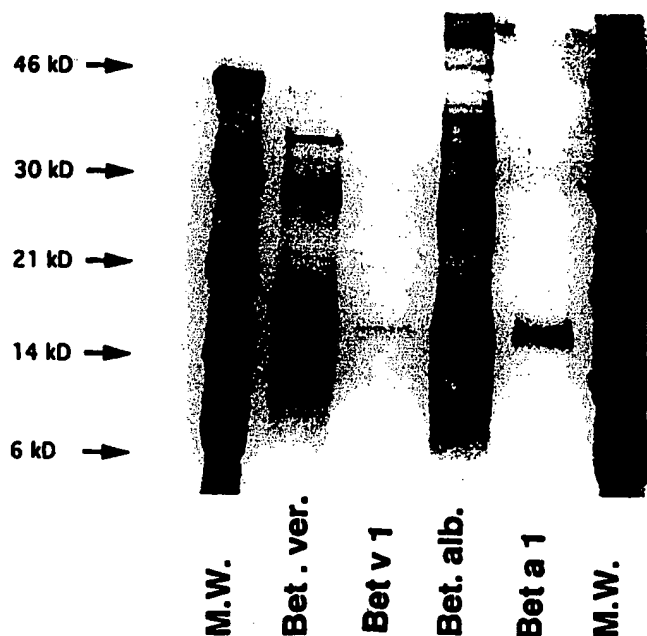
FIG. 1. Silver stain of an SDS-polyacrylamide gel containing natural Bet v 1 purified from *B. alba* and *B. pendula* pollen by immunoaffinity with mAb 7–coupled Sepharose. *M.W.*, Molecular weight marker; *Bet. ver.*, 40 µg of birch pollen extract; *Bet v 1*, 0.8 µg of material eluted from *B. verrucosa* extract; *Bet. alb.*, 400 µg of birch pollen extract; *Bet a 1*, 8 µg of material eluted from *B. alba* extract.

centrifugation at 4000 g, the supernatants were transferred into new tubes for measurement of histamine.

For the inhibition assays, 50 µl of the Bet v 1 preparation was preincubated for 90 minutes at 37° C with 50 µl of a mixture of nine mouse mAbs (mAbs 1, 2, 4, 7, 8, 9, 10, 11, and 15; each present at 150 µg/ml in the preincubation mixture). Group 1 and group 2 mAbs were included in the mixture to mimic a polyclonal serum. For control purposes, an mAb without specificity for Bet v 1 adjusted to the same total protein concentration was used for preincubation. Four hundred microliters of the cell suspension was then added to the preincubated Bet v 1, and the histamine release assay proceeded as described above. Each experiment was done in triplicate. The histamine content was quantified by an automated fluorometric method.[26] Results were expressed as the percentage of histamine content as follows: $100(a - b) \div (c - b)$, where a = mean of the fluorometric readings for experimental supernatant, b = mean for spontaneous histamine release, and c = mean for total histamine. Inhibition of histamine release was expressed as $\{1 - (HRp \div HRc)\} \times 100$, where HRp and HRc are the percents of histamine release observed when the allergen was preincubated with the mouse mAbs or with Tris-ACM buffer, respectively.

RESULTS

Generation and characterization of mouse mAbs specific for Bet v 1

Immunization of BALB/c mice with pollen grains from *B. verrucosa* yielded 85 hybridomas producing Bet v 1–specific mAbs. A large proportion of the birch pollen–specific hybrid mas (>90%) produced Bet v 1–specific antibodies. The specificity of the mAbs was established in direct ELISA and confirmed by immuno-

precipitation of Bet v 1 from ^{125}I-labeled birch pollen extract. Monoclonal antibody 7 was used to purify natural Bet v 1 from extracts of *B. alba* and *B. pendula* pollen to homogeneity in a single-step immunoaffinity purification (Fig. 1). The mAbs also reacted with nitrocellulose-blotted natural and recombinant Bet v 1 (Fig. 2, A). A sandwich assay, based on mAb 8 and mAb 10, was established for the fluid-phase measurements of Bet v 1 concentrations, indicating that mAbs 8 and 10 most likely bound to nonidentical epitopes of Bet v 1 (data not shown).

Bet v 1–specific mAbs cross-react with Bet v 1 homologous plant allergens

Cross-reactivity of serum IgE from patients allergic to birch pollen with Bet v 1 homologous allergens from different tree pollens, fruits, and vegetables explains clinical symptoms on contact with various tree pollens and on ingestion of plant-derived food.[27] In Fig. 2, B, the cross-reactivity of 18 selected mAbs with nitrocellulose-blotted Bet v 1 homologous allergens from alder pollen (Aln g 1),[12] hazel pollen (Cor a 1),[13] hazelnut,[15] and apple (Mal d 1)[14] is demonstrated. A high degree of cross-reactivity with the pollen-derived Bet v 1 homologs was observed; whereas only about half of the mAbs cross-reacted with the maj r hazelnut allergen, and only f ur mAbs (mAbs 2, 9, 12, and 13) and Bip 1[23] reacted with the maj r apple allergen, Mal d 1. The different reactivity of the mAbs with hazel pollen extract versus
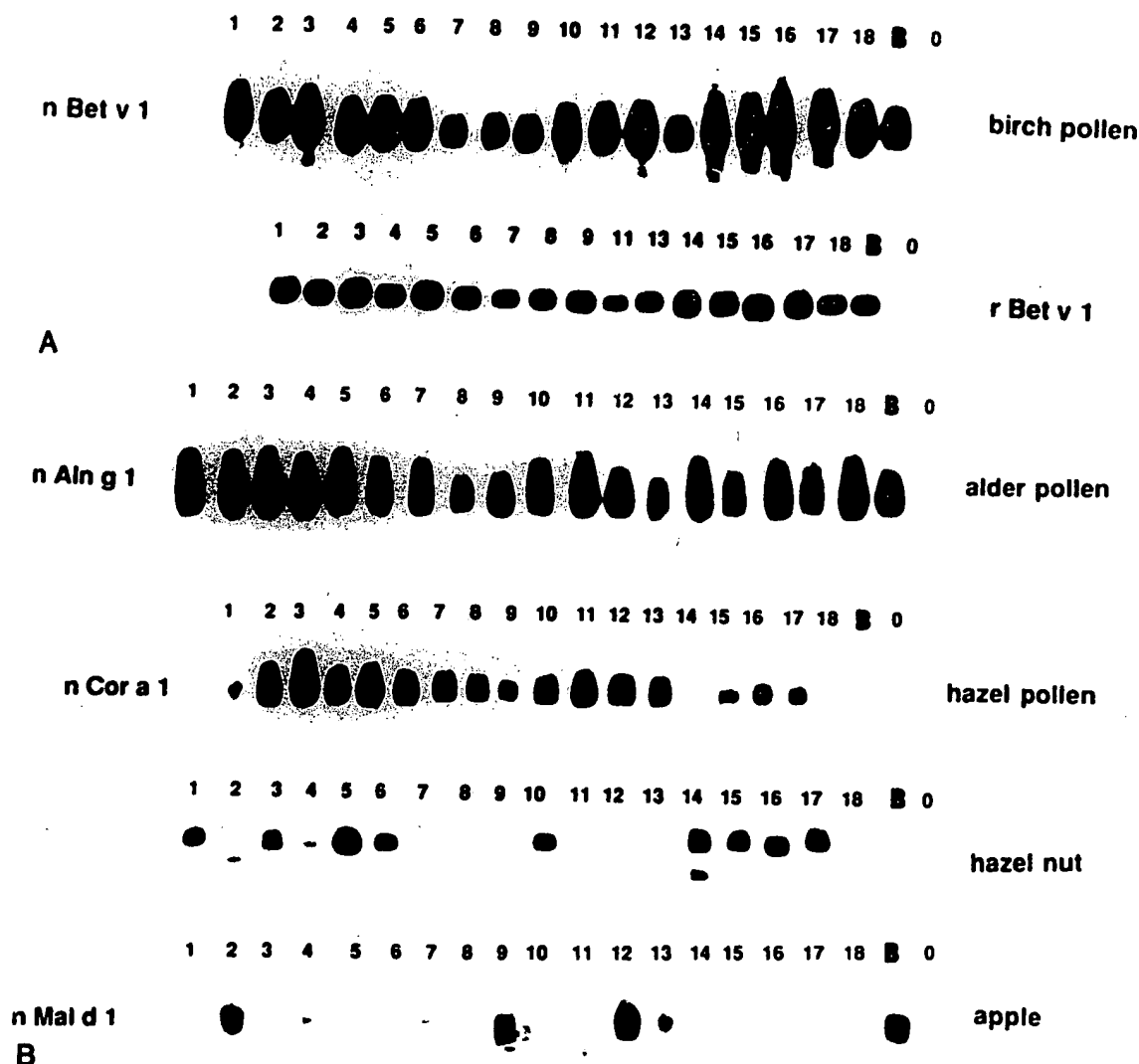
FIG. 2. Binding of mouse mAbs to nitrocellulose-blotted natural and recombinant Bet v 1. A, Approximately 50 ng/cm of nitrocellulose-blotted natural Bet v 1 *(upper row)* and recombinant Bet v 1 *(lower row)* were incubated with Bet v 1-specific mAbs (mAbs 1 to 18, *lanes 1 to 18)*, a Bet v 1-specific control antibody, Bip 1 *(lane B)*, and buffer without addition of mouse mAbs (negative control, *lane 0)*. B, Cross-reactivity of Bet v 1-specific mAbs with homologous plant allergens. Nitrocellulose-blotted extracts from alder pollen, hazel pollen, hazelnuts, and apple were incubated with Bet v 1-specific mAbs in the same order as in A. Bound IgG was detected with an ^{125}I-labeled sheep anti-mouse antiserum.
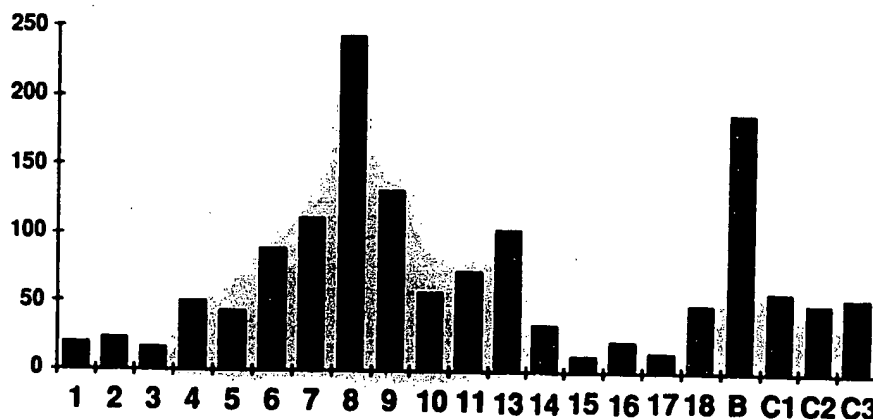
hazelnut extracts indicates that different isoforms of Cor a 1 may be expressed in pollen and somatic tissues. When tested in ELISA, all 18 mAbs cross-reacted with alder pollen extract (data not shown).

Cross-inhibition of the mAbs in ELISA defines two groups of Bet v 1-specific mAbs

In an attempt to characterize B-cell epitopes f the mAbs by cross-competition experiments, all 85 Bet v 1-specific mAbs were tested for their capacity t inhibit the binding of 35 purified and peroxidase-labeled mAbs by ELISA. The results were confirmed by using the 35

purified unlabeled mAbs as competitors. Table I shows a summary of the data obtained with representative antibodies. The mAbs could be segregated into two major groups: group I mAbs bound strongly to Bet v 1 coated on plastic and defined epitopes (A to D) (Table I); group II mAbs bound weakly to Bet v 1 in direct ELISA but very efficiently in sandwich ELISA and defined epitopes (E to G) (Table I). The difference between the tw groups of mAbs might explain the lack of inhibition by group II mAbs. Group II mAbs were easily c mpeted by group I mAbs in direct ELISA. Six group I mAbs, mAbs 1, 2, and 10 (as well as mAb 5 and mAbs 14 t 18, data
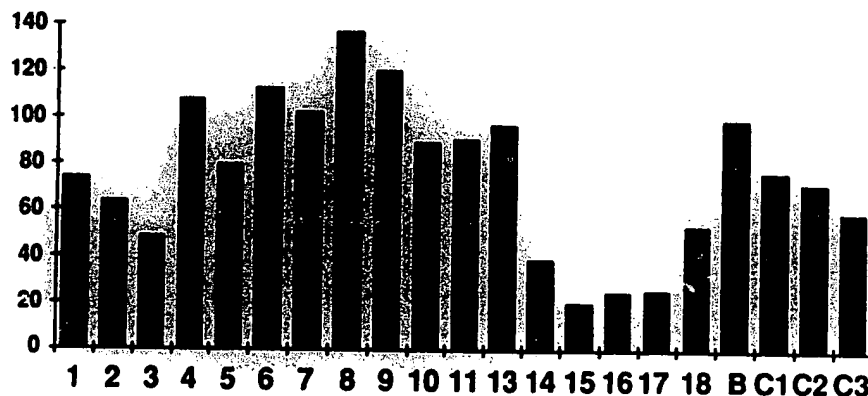
patient 1

patient 2

FIG. 3. Murine anti-Bet v 1 mAbs modulate IgE recognition of nitrocellulose-blotted Bet v 1. Nitrocellulose-blotted recombinant Bet v 1 was preincubated with mAbs 1 to 11 and mAbs 13 to 18, *(1 to 11 and 13 to 18)* with Bip 1 *(B)*, with serum from a nonallergic individual diluted 1:5 and undiluted *(C1 and C2,* respectively), or with buffer *(C3)*. Blots were then incubated with serum IgE from two patients allergic to Bet v 1 (patient 1; patient 2). IgE was detected with ^{125}I-labeled monoclonal anti-human IgE antibodies, visualized by autoradiography, and quantified by densitometric analysis. Values displayed at the y axis correspond to amounts of bound IgE antibodies.

not shown) define one epitopic region according to their high level of cross-inhibition. The group II mAbs, including mAbs 7, 8, and 9 (as well as mAbs 6 and 12, data not shown) identify several distinct epitopes.

Characterization of epitopes of group I mAbs with synthetic overlapping Bet v 1 peptides

Monoclonal antibodies 1 to 18 were tested for reactivity with synthetic Bet v 1 dodecapeptides with an overlap of nine amino acids, spanning the complete Bet v 1 sequence, which were coupled t ELISA plates. Most of the mAbs did not bind to the synthetic overlapping peptides, indicating that they bind to discontinuous epitopes. Four mAbs (mAbs 1, 2, 14, and 16), all

belonging to group I as described above, reacted with the same three synthetic peptides (p17, p18, and p19), which constitute a continuous Bet v 1 epitope in the region of amino acids 49 to 66. The epitope mapping was confirmed by using peptides that were dot-blotted to nitrocellulose (data not shown).

Bet v 1-specific mAbs modulate IgE binding of patients with allergy

To investigate the effects of mouse monoclonal anti-Bet v 1 antibodies on the binding to Bet v 1 f IgE from patients allergic to tree pollen, competition experiments were done. Nitrocellulose strips containing equal amounts (10 ng/cm) of recombinant Bet v 1 were

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TABLE I. Cr ss-competiti n f th anti-Bet v 1 mAbs

Inhibitor	Peroxidase-Labeled mAb									Epitope
	mAb 1	mAb 10	mAb 2	mAb 11	mAb 3	mAb 4	mAb 7	mAb 8	mAb 9	
mAb 1	100	95	82	60	67	—	98	96	84	
mAb 10	96	100	100	60	72	—	—	32	30	A
mAb 2	18	87	99	45	50	—	76	78	72	
mAb 11	—	—	45	100	55	39	77	72	72	B
mAb 3	—	—	—	—	92	0	56	59	74	C
mAb 4	—	—	38	50	47	97	73	67	73	D
mAb 7	—	—	—	—	—	—	97	—	26	E
mAb 8	—	—	22	—	—	—	—	84	—	F
mAb 9	—	—	—	—	20	—	—	—	93	G

ELISA cross-competition experiments allowed the definition of two groups of Bet v 1-specific antibodies (groups I and II). Results are expressed as percent of binding inhibition; minus sign (−) indicates that no significant inhibition (inhibition <15%) was observed. The cross-competition allowed the definition of seven epitopic areas on Bet v 1 (A to G), which were assembled into two groups: group I (epitopes A to D) and Group II (epitopes E to G).

preincubated with an excess of mAb. Because of the extremely low concentration of serum IgE (10 to 500 ng/ml), sera were diluted only 1:5 to visualize differences in the IgE binding. Fig. 3 shows the densitometric analysis of autoradiographs documenting the effects of preincubation of Bet v 1 with different mAbs and controls (x axis) on the IgE binding tested in two representative patients (Fig. 3, A, patient 1; B, patient 2). Although some of the antibodies had little effect on the patients' IgE binding to Bet v 1 or different effects, depending on the serum tested, two series of antibodies strongly modulated IgE binding. Monoclonal antibodies 3, 14, 15, 16, and 17 (all belonging to group I) inhibited IgE binding to nitrocellulose-blotted Bet v 1 of both sera up to 80%; whereas mAbs 6, 7, 8, 9, and 13 (belonging to group II) enhanced IgE binding to Bet v 1 up to fourfold. A previously described Bet v 1-specific mAb, Bip 1,[23] (Fig. 3, patient 1) enhanced IgE binding to Bet v 1. Preincubation with mAbs or sera without specificity for Bet v 1 (data not shown) and sera from nonallergic individuals (19 and 20) had no effect on IgE binding.

To exclude that the observed effects on the Bet v 1-IgE interaction are due to renaturation of a denatured allergen, the same experiment was done with dot-blotted, undenatured recombinant Bet v 1 by using 56 sera and mAbs 14, 15, 16, 17, and 18. The recombinant Bet v 1 used for this experiment was shown to be correctly folded by circular dichroism analysis. Fig. 4, A, shows the outcome of this experiment. Monoclonal antibody 14 reduced IgE binding to Bet v 1 in 75% of the sera; mAb 15 inhibited IgE binding in 79%, mAb 16 in 69%, mAb 17 in 86%, and mAb 18 in 73%. Although mAbs 14 to 18 belonged to group I, they were able to enhance IgE binding to Bet v 1 in certain sera (e.g., sera 18, 26, 43, and 51) up to twofold. Additional mAbs (including group II mAbs 8 and 9) were tested for their capacity t modulate IgE binding to Bet v 1 (Fig. 4, B). Enhancement of IgE binding t Bet v 1 (e.g., mAb 9: sera B and C), inhibition of IgE binding to Bet v 1 (e.g., mAb 9: serum A), r no effect n IgE binding to Bet v 1 (e.g.,

mAbs 8 and 9: serum D) was observed, depending on the serum used. These data indicate that the Bet v 1-specific mAbs are able to modulate IgE binding, depending on the presence of certain IgE specificities in each serum.

A mouse mAb without specificity for Bet v 1 (Fig. 4, B, co) did not modulate IgE binding.

A mixture of nine mAbs inhibits Bet v 1–induced histamine release

To address the biologic consequence of in vitro competition of IgE binding to Bet v 1, we have investigated whether a mixture of mAbs might be able to influence the Bet v 1–induced degranulation of basophils from patients with allergy. Fig. 5 shows that preincubation of Bet v 1 with a mixture of nine mAbs (mAbs 1, 2, 4, 7, 8, 9, 10, 11, and 15) inhibited Bet v 1–induced histamine release up to 90%. An mAb without specificity for Bet v 1, adjusted to the same protein concentration as the Bet v 1 mAb mixture, did not significantly affect Bet v 1–induced histamine release (buffer control, 64% histamine release; control mAb, 67% histamine release). A control experiment in which the patients' basophils were incubated with the mixture of the nine mAbs without addition of Bet v 1 demonstrated a lack of toxic effects of the mAb mixture.

DISCUSSION

The cross-linking of effector cell–bound specific IgE antibodies by allergens represents the key event that leads to the liberation of biologic mediators, such as histamine, from basophils and mast cells, which then causes allergic rhinitis, conjunctivitis, and asthma. The definition of IgE epitopes is hence relevant to devise specific forms of therapy, which are based on interfering with the allergen-IgE interaction. If continuous (sequential) IgE epitopes of major allergens can be defined, it may be possible to dissect allergens int IgE-binding hapt ns, which bind IgE but do not cross-link effect r cell–bound IgE antibodies as blocking tools.[17] In the
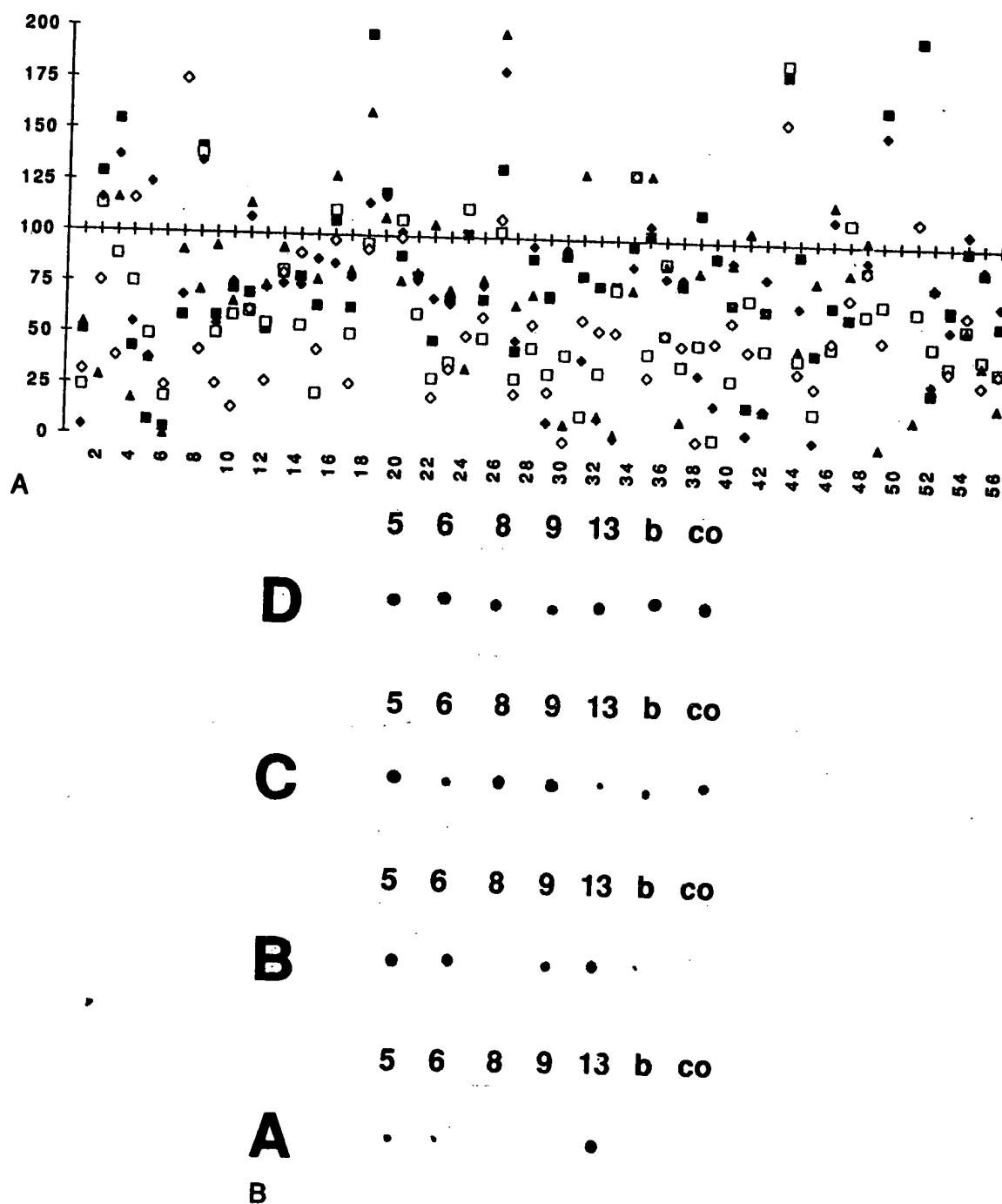
FIG. 4. Modulation of IgE binding to dot-blotted Bet v 1 by mAbs. A, Five monoclonal anti-Bet v 1 antibodies (mAbs 14, 15, 16, 17, and 18) and buffer without addition of mAb were tested for their ability to influence IgE binding of sera from 56 patients allergic to birch pollen to dot-blotted Bet v 1 (each second serum is numbered on bottom of x axis). Bound IgE was detected with ^{125}I-labeled anti-human IgE antibodies and quantified by gamma counting. The line at 100 corresponds to IgE binding when Bet v 1 was preincubated with buffer only. Symbols on the y axis show the percentage of binding to Bet v 1 on preincubation with individual mAbs in each patient. In sera 14, 19, 43, 50, and 51, the absence of symbols indicates an enhancement of IgE binding of more than twofold. *Filled squares,* mAb 14; *open squares,* mAb 15; *filled diamonds,* mAb 16; *open diamonds,* mAb 17; *filled triangles,* mAb 18. B, Experiment similar to that in A, performed with mAbs 5, 6, 8, 9, and 13; buffer *(b);* and a control antibody without specificity for Bet v 1 *(co)* by using four additional representative sera (A to D). IgE bound to dotted Bet v 1 was detected with ^{125}I-labeled anti-human IgE and visualized by autoradiography.
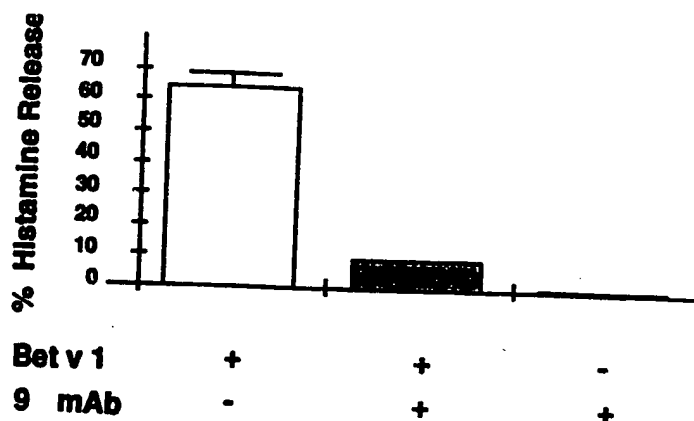
FIG. 5. A mixture of Bet v 1-specific mAbs inhibits Bet v 1-induced histamine release. Natural Bet v 1 was used at a concentration of 10^{-10} gm/ml. Percentage of histamine release as mean value from triplicate determinations, measured after preincubation of Bet v 1 with the mAbs or without preincubation, is displayed on the y axis. As a negative control, the effect of mAbs on basophil histamine release without addition of Bet v 1 is also displayed.

case of the major birch pollen allergen Bet v 1 and related allergens, which are present in various tree pollens, fruits, and vegetables, no continuous epitopes have yet been defined. Neither Bet v 1-derived synthetic peptides, proteolytic fragments, nor recombinant Bet v 1 fragments bound IgE; and it was hence assumed that Bet v 1 IgE epitopes belong to the discontinuous (conformational) type. Conformational epitopes were in fact described for Bet v 3, a calcium-binding birch pollen allergen, in which the presence of protein-bound calcium was required to assemble the IgE epitopes.[28]

In this study a panel of 85 murine monoclonal anti-Bet v 1 antibodies was characterized for their capacity to interfere with allergic patients' IgE binding to Bet v 1. Monoclonal antibodies were characterized according to their cross-reactivity with Bet v 1 homologous plant allergens. Like IgE antibodies from patients with allergy, most mAbs displayed extensive cross-reactivity with the major allergens of alder pollen, Aln g 1,[12] hazel pollen, Cor a 1,[13] hazelnut,[15] and apple, Mal d 1.[14]

Cross-inhibition experiments between the mAbs led to their classification into two major groups: group I mAbs strongly recognized Bet v 1 in a direct ELISA, whereas group II mAbs bound weakly in the direct assay. These mAbs bound strongly to Bet v 1 presented in a sandwich ELISA. Group I mAbs inhibited the binding of group II mAbs to Bet v 1 in direct ELISA, whereas the reciprocal was not true. Similar single-way inhibition was reported previously for two mAbs raised against Bet v 1.[29] When tested for interference with binding of human IgE to Bet v 1, mAbs 1, 2, 3, 14, 15, 16, and 17 (all from group I) inhibited IgE binding up to 99% in certain patients. Most of the mAbs that competed with patients' IgE binding did n t bind to dodecapeptides derived from Bet v 1. Only three of the competing mAbs (mAbs 1, 14, and 16), though from different hybridomas, recog-

nized the same epitopic region (amino acids 49 to 66) of Bet v 1, which also comprises an area of predicted high antigenic index, perhaps representing part of a discontinuous IgE epitope on Bet v 1.

The inhibition of IgE binding to Bet v 1 by several mAbs can be explained by conformational changes of Bet v 1 because of induced fit or by blocking of IgE binding because of use of the same epitopes by mAbs and human IgE. The consistent enhancement of IgE binding by certain mAbs-particularly mAbs 6, 7, 8, and 9 belonging to the second group-and by a previously described Bet v 1-specific mAb, Bip 1,[23] may be due to conformational changes of Bet v 1 on mAb binding. One explanation for the enhancement would be that some of the mAbs renature nitrocellulose-blotted Bet v 1. The enhancement of IgE binding was, however, also observed with undenatured, dot-blotted Bet v 1, which was checked for proper folding by circular dichroism analysis before. It should also be pointed out that in certain of the 56 sera tested in Fig. 4,A, significant enhancement of IgE binding to Bet v 1 was observed with antibodies that blocked IgE binding in most other sera.

It is hence conceivable that some of the mAbs stabilize Bet v 1 conformations, most likely by induced fit, so that IgE epitopes are more accessible. This may then allow binding of certain species of Bet v 1-specific IgE to Bet v 1. In fact, changes of protein conformations by mAbs have been reported earlier for cytochrome c[30] and hemoglobin.[31] In the case of hemoglobin the binding of mAbs leads to changes in the affinity for oxygen without directly involving the portions responsible f r oxygen binding.

A stabilization of such Bet v 1 states might ither reveal IgE epitopes r stabilize epitopes for high-affinity binding. The enhancement of antibody binding to a given antigen by other antibodies that bind t different epitopes may perhaps even represent a general mecha-

nism for the amplification of humoral immune responses against antigens with discontinuous epitopes, such as Bet v 1. In fact, human Bet v 1-specific IgG antibodies with similar activity could be isolated from a patient allergic to pollen,[32] and similar phenomena were reported for antibodies binding to influenza viruses.[33] Structural analysis of the Bet v 1 antibody interactions (e.g., x-ray crystallography and nuclear magnetic resonance analysis) will, however, be required to finally clarify the mechanism of enhancement.

The strong inhibition of IgE binding by certain group I mAbs (up to 99%), which was observed in a large number of sera, suggests that the use of blocking antibodies be considered for local therapy of Bet v 1-induced allergy. In fact, it was also demonstrated that the Bet v 1-induced histamine release from patients' basophils could be inhibited with the mAbs. However, when considering the use of Bet v 1-specific antibodies or antibody fragments for therapy, a careful selection of the antibodies with regard to their inhibitory and enhancing activities is required. Nevertheless, it is likely that humanized mAbs or recombinant antibody fragments might be useful for interference with the Bet v 1-IgE interaction to prevent allergen-induced mediator release in the mucosa of the lungs and nose and in the conjunctiva.

REFERENCES

1. Ishizaki L, Koizoumi R, Ikemori R. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. Ann Allergy 1987;58:265-70.
2. Noon L. Prophylactic inoculation for hayfever. Lancet 1911;1:1572-3.
3. Thompson RA. The current status of allergen immunotherapy (hyposensitisation). Allergy 1989;44:369-79.
4. Coleman JW, Helm BA, Stanworth DR, Gould HJ. Inhibition of mast cell sensitization in vitro by a human immunoglobulin epsilon-chain fragment synthesized in *Escherichia coli*. Eur J Immunol 1985;15:966-9.
5. Chretien I, Pene J, Briere F, De Waal Malefijt R, Rousset F, De Vries JE. Regulation of human IgE synthesis. I. Human IgE synthesis in vitro is determined by the reciprocal antagonistic effects of interleukin 4 and interferon-gamma. Eur J Immunol 1990;20:243-51.
6. Valenta R, Kraft D. Recombinant allergens for diagnosis and therapy of allergic diseases. Curr Opin Immunol 1995;7:751-6.
7. Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, et al. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. Allergy 1989;44:385-95.
8. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, et al. The gene coding for the major birch pollen allergen, Bet v 1, is highly homologous to a pea disease resistance response gene. EMBO J 1989;8:1935-8.
9. Bufe A, Spangfort MD, Kahlert H, Schlaak M, Becker WM. Major birch pollen allergen Bet v 1 shows ribonuclease activity. Planta 1996. In press.
10. Rohac M, Birkner T, Reimitzer I, Bohle B, Steiner R, Breitenbach M, et al. The immunological relationship of epitopes on major tree pollen allergens. Mol Immunol 1991;28:897-906.
11. Valenta R, Breiteneder H, Pettenburger K, Breitenbach M, Rumpold H, Kraft D, et al. Homology of the major birch pollen allergen, Bet v 1, with the major pollen allergens of alder, hazel, and hornbeam at the nucleic acid level as determined by cross-hybridization. J Allergy Clin Immunol 1991;87:677-82.
12. Breiteneder H, Ferreira F, Reikerstorfer A, Duchène M, Valenta R, Hoffmann-Sommergruber K, et al. Complementary DNA cloning and expression in *Escherichia coli* of Aln g 1, the major allergen in pollen of alder (*Alnus glutinosa*). J Allergy Clin Immunol 1992;90:909-17.
13. Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, et al. Four recombinant isoforms of Cor a 1, the major allergen of hazel pollen, show different IgE binding properties. Eur J Biochem 1993;212:355-62.
14. Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, et al. Common epitopes of birch pollen and apples-studies by Western and Northern blot. J Allergy Clin Immunol 1991;88:588-94.
15. Hirschwehr R, Valenta R, Ebner C, Ferreira F, Sperr WR, Valent P, et al. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. J Allergy Clin Immunol 1992;90:927-36.
16. Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, et al. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). J Allergy Clin Immunol 1995;95:962-9.
17. Ball T, Vrtala S, Sperr WS, Valent P, Susani M, Kraft D, et al. Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. Dissection of the allergic effector reaction. J Biol Chem 1994;269:28323-8.
18. Wilson MB, Nakane PK. In: Knapp W, Holubar K, Wick G, editors. Immunofluorescence and related staining technics. Amsterdam: Elsevier, 1978.
19. Vallier P, DeChamp C, Valenta R, Vial O, Deviller P. Purification and characterization of an allergen from celery immunochemically related to an allergen present in several other plant species. Identification as a profilin. Clin Exp Allergy 1992;22:774-82.
20. Ferreira F, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, et al. Purification and characterization of recombinant Bet v 1, the major birch pollen allergen. Immunological equivalence to natural Bet v 1. J Biol Chem 1993;268:19574-80.
21. Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high molarity Tris buffer system without urea. Anal Biochem 1986;155:83-8.
22. Towbin H, Staehelin T, Gordon J. Electrophoretic transfers of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350-4.
23. Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D, et al. Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major birch pollen allergen Bet v 1. Int Arch Allergy Appl Immunol 1989;90:54-60.
24. Valenta R, Duchène M, Vrtala S, Birkner T, Ebner C, Hirschwehr R, et al. Recombinant allergens for immunoblot diagnosis of tree pollen allergy. J Allergy Clin Immunol 1991;88:889-94.
25. May CD, Lyman M, Alberto R, Cheng J. Procedures for immunochemical study of histamine release from leucocytes with small volume of blood. J Allergy 1970;46:12-20.
26. Lebel B. A high-sampling rate automated continuous-flow fluorometric technique for the analysis of nanogram levels of histamine in biological samples. Anal Biochem 1983;133:16-29.
27. Valenta R, Kraft D. Type I allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. J Allergy Clin Immunol 1996;97:893-5.
28. Seiberler S, Scheiner O, Kraft D, Lonsdale D, Valenta R. Characterization of a birch pollen allergen, Bet v 3, representing a novel class of Ca²⁺ binding proteins: specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca²⁺. EMBO J 1994;13:3481-6.
29. Mare SI, Boutin Y, Vrancken ER, Hebert J. Mapping of Bet v 1 epitopes by using murine monoclonal antibodies. Int Arch Allergy Appl Immunol 1990;92:226-32.
30. Collawn JF, Wallace CJA, Proudfoot AEI, Paterson Y. Monoclonal

antibodies as probes of conformational changes in protein-engineered cytochrome c. J Biol Chem 1988;263:8625-34.

31. Dean J, Schechter AN. Conformation-specific antibodies to the a chain COOH terminus of hemoglobin A₀. J Biol Chem 1979;254:9185-93.

32. Visco V, Dolecek C, Denépoux S, Le Mao J, Guret C, Rousset F, et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. J Immunol 1996;157:956-62.

33. Lubeck M, Gerhard W. Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. Virology 1982;118:1-7.